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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
We investigated the occurrence, origin and have begun to explore the biological function of elevated reactive oxygen species (ROS) in breast carcinoma cell lines, particularly with regard to aberrant expression of Nox isoforms. We have initially surveyed 7 breast carcinoma cell lines including BT-20, BT-474, MCF7, MDA-MB-231, SK-BR-3, T-47D, and ZR-75-1, available through ATCC. These include lines developed from both primary and metastatic tumors. In addition, we surveyed three control cells lines, MCF10A, MCF12A, and 184A1 derived from either fibrocystic disease or breast reduction. We investigated the generation of the H_2O_2 and O_2^-. While O_2^- levels appeared to remain unchanged, H_2O_2 levels increased significantly over control cell lines in several of the tumor cell lines. Interestingly, the addition of estradiol to the estradiol dependent MCF7 cell line greatly increased H_2O_2 production. Increased H_2O_2 levels corresponded with increased tumorigenicity based on literature searches. Analysis of mRNA expression of the Nox isoforms revealed that the above cell lines contain the message for Nox4 and Nox5. We have recently tested the cell lines for Nox4 and Nox5 protein expression using Nox4 and Nox5 antibodies developed in our lab. Nox4 expression is very apparent in the cells. Nox5 protein expression needs further exploration.

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INTRODUCTION

While non-transformed cells respond to growth factors/cytokines with the regulated production of ROS, tumor cells produce ROS in an uncontrolled manner (Szatrowski and Nathan 1991). Ras-transformed NIH 3T3 cells generated increased O_2^-, and inhibition of O_2^- generation caused reversion to a non-transformed phenotype (Irani *et al.* 1997). O_2^- is also implicated in maintenance of transformation in melanoma, breast carcinoma, fibrosarcoma, and virally transformed tumor cells: decreased MnSOD (and by inference, increased O_2^-) is seen in these cancers/cell lines. MnSOD is encoded on chromosome 6q25 which is frequently lost in melanoma, and overexpression of MnSOD in melanoma (Church *et al.* 1993) and other cancers (Yan *et al.* 1996; Fernandez-Pol *et al.* 1982) suppressed the transformed phenotype. In addition, Nox1 overexpressing NIH 3T3 cells which showed high levels of O_2^- and H_2O_2 and exhibited increased growth rates showed a strongly transformed phenotype forming foci and appearing to lose contact inhibition. Cells also exhibited anchorage-independent growth (Suh *et al.* 1999). The transformed phenotype was reverted when these cells were transfected with catalase to remove the excess hydrogen peroxide (Arnold *et al.* 2001). Thus, elevation of ROS either by overproduction or decreased metabolism is correlated with cell transformation and cancer. Therefore, the purpose of this concept grant was to begin to explore the role of ROS in breast cancer, particularly with respect to Nox isoforms.

BODY

A. Description of Cell Lines: From American Type Culture Collection ATCC CellLines and Hybridomas 8th Edition 1994. All cell lines are human and epithelial-like.
MCF 10A: Established from mammary tissue from a patient with fibrocystic disease.
MCF 12A: A non-tumorigenic epithelial cell line of normal mammary tissue.
184A1: Established from normal breast tissue obtained at reduction mammoplasty. These cells were chemically transformed with benzo(a)pyrene.
BT20: Established from breast carcinoma. Cells form grade II adenocarcinomas in nude mice.
BT474: Established from a solid, invasive ductal carcinoma of the breast. The cell line is reportedly tumorigenic in nude mice.
MCF7: Cells are from a pleural effusion of a breast adenocarcinoma. They have retained their ability to process estradiol via estrogen receptors.
MDA MB 231: Cells are from a pleural effusion of a breast adenocarcinoma. The cells form poorly differentiated grade III tumors in nude mice.
SK BR 3: Cells are from a malignant pleural effusion of a breast adenocarcinoma and produce poorly differentiated grade III tumors in nude mice.
T47D: Cells were from a pleural effusion from an infiltrating ductal carcinoma of the breast.
ZR 75 1: Derived from a malignant ascitic effusion from an infiltrating ductal carcinoma. Cells possess receptors for estrogen and other steroid hormones.

B. *RT-PCR of total RNA from breast cell lines*: Total RNA was harvested from BT-20, BT-474, MCF7, MDA-MB-231, SK-BR-3, T-47D, and ZR-75-1 breast cancer cell lines and MCF10A, and 184A, control breast cell lines. Following reverse transcription of the RNA, PCR was

performed using primers sets specific for Nox1, gp91, Nox3, Nox4, Nox5, duox1 and duox2 (duox, dual oxidase). These primer sets are currently in use in the lab and detailed desciption of them can be found in Cheng et al. 2001. Nox4 message was present in all cell lines tested, as was Nox5. Weak bands corresponding to duox1 and duox 2 were also observed in some of the cell lines (data not shown).

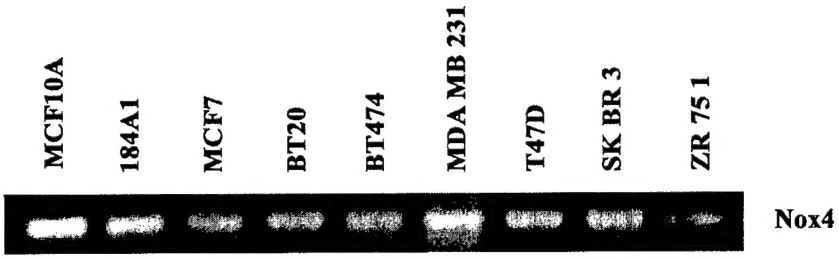

Figure 1A

Fiugre 1. PCR of cDNA from cell lines as detailed in figure. Briefly, Nox4 primers (5'-TCCATTTACCCTCACAA TGTGT-3' and 3'-AGAGGAACACGACAAT CAGCCTTAG-3') and Nox5 primers (5'-CTCATTGTCACACTCCTCGACAGC-3' and 5'-ATCAAGCGGCCCCCTTTTTTTCAC-3') were used at a concentration of 280 nM. PCR parameters were 95°C for 45 sec, 66°C or 63.5°C for 45 sec, 72°C for 2 min, 35 cycles after denaturing for 2 min at 95°C. Nox4 results are shown in Figure 1A. Nox5 results are shown in Figure 1B.

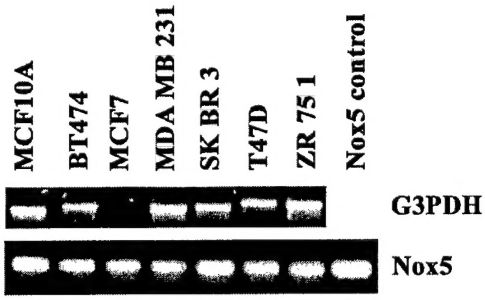

Figure 1B

C. *DHE assay of cell lines.* A number of the cell lines were digested, treated with 10 μM dihydroethiduim (DHE) for 1 hr then analyzed by FACS machine for fluorescence of the ethidium (Table 1). The DHE is taken up by the cells, reduced by superoxide to ethidium, then the ethidium binds to the DNA in the cell and fluoresces. This method measures intracellular levels of superoxide. There was no significant change in the superoxide levels of any of the cell lines tested. Error represents the difference between two determinations.

Cell Line	Mean Fluorescence
MCF10A	122 ± 14
BT474	102 ± 4
SKBR3	120 ± 5
MDAMB231	81 ± 26
ZR751	89 ± 26
T47D	81 ± 12

Table 1

D. *DCFDA assay of cell lines.* All of the cell lines were digested, treated with 2 μM dichlorofluorescein diacetate (DCFDA) for 1 hr then analyzed by FACS machine for fluorescence of the dichlorofluorescein (DCF) (Table 2). The DCFDA is taken up by the cells and converted to DCF which reacts with hydrogen peroxide and fluoresces. This method measures intracellular levels of hydrogen peroxide. As shown in Table 2, a number of the cell lines tested showed increased hydrogen peroxide levels. Error represents the standard error of the mean (4-14 samples). A search of relevant literature indicates a correlation between increased

hydrogen peroxide levels and increased tumorigenicity (American Type Culture Collection, 1994 (see above); Dickson *et al.* 1986; Madsen and Briand, 1990).

Cell Line	Mean Fluorescence
MCF12A	18 ± 1
MCF10A	26 ± 4
184A1	30 ± 6
MCF7	22 ± 3
BT20	37 ± 6
SKBR3	40 ± 6
T47D	41 ± 5
BT474	48 ± 11
ZR751	63 ± 15
MDAMB231	66 ± 11

Table 2

E. *Estradiol treatment of several breast cell lines.* MCF10A, MCF7, or ZR751 cells were treated for 4 hrs with vehicle or 10 nM estradiol in 0.001 % ethanol in RPMI media. Following treatment, cells were harvested by trypsin digestion, then incubated with 2 μM DCFDA in the absence or presence of 10 nM estradiol. Following a 1 hr incubation at room temperature, fluorescence was measured on a FACS machine The mean fluorescence is reported in Table 3. Error represents the difference between two determinations. Estradiol had little effect on control (MCF10A) cells, however hydrogen peroxide levels were increased 2.5 fold in MCF7 cells treated with estradiol. Interestingly, tumor incidence in nude mice injected with MCF 7 is greatly enhanced when the mice are fed estradiol (Dickson et al. 1986).

Cell Line	Mean Fluorescence (control)	Mean Fluorescence (estradiol)
MCF10A	24 ± 11	13 ± 2
MCF7	29 ± 4	73 ± 9

Table 3

F. *Nox4 protein levels.* All the cell lines except 184A1 were harvested for protein and the lysates run on a 10% polyacrylamid gel. The proteins were transferred to nitrocellulose and the membrane blotted with antibodies that recognize Nox4. All cell lines tested contained nox4 protein. The MCF12A sample ran faster through the gel, possibly due to protein degradation. HEK 293 cells (human embryonic kidney) were run as a positive control.

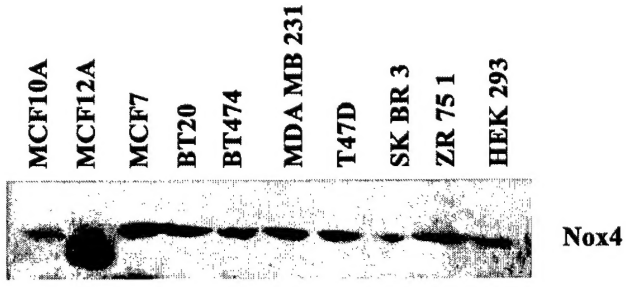

Figure 2

Figure 2. Cell lysates were harvested wth 5 mM $CaCl_2$ in phosphate buffered saline. Following pelleting, the cells were resuspended in HANKS buffered saline containing protease inhibitors. The lysates were sonicated briefly on ice followed by determination of protein concentration. 40 μg protein was added to each well of a 10 % polyacrylamide gel. Following electrophoresis, the protein was transferred to a nitrocellulose membrane. The membrane was blocked using 5 % milk in Tris Buffered Saline containing 0.05 % Tween 20. The Nox4 antibody (1:2000) was incubated on the blot in this same buffer overnight at room temperature. Following washing, the anti rabbit secondary antibody (1:10,000) was incubated on the blot for 3 hrs then the proteins visuallized.

G. *Nox5 protein levels.* The cell lines were also harvested for Nox5 protein level evaluation. The Nox5 antibody has been newly developed in our lab, designed to recognize a region in the soluble C-terminal portion of the protein. Nox5 has three splice varients, ranging in size from 65kD to 192 kD. Western blot analysis of these cells did not reveal any bands at 65 kD. However, there were two faint bands at 116 kD and 192 kD possibly corresponding to the two other isforms (data not shown). Future work will involve more detailed analysis of these bands to determine if they are indeed Nox5.

H. Future plans. Future plans include investigating the biochemical and functional relevance of Nox overexpression. On possible approach includes using antisense transfection to decrease the expression of specific isoforms. In addition, in collaboration with Dr. Cindy Farach-Carson, we are currently designing Nox ribozymes to knockout Nox4 or Nox5 expression in these cell lines. In addition, we have determined that the Nox4 antibody described above is useful in immunohistochemistry. We will use this antibody in collaboration with the Department of Pathology and we will determine if Nox4 protein levels differ in breast vs breast cancer tissue.

KEY RESEARCH ACCOMPLISHMENTS

1. Nox4 and Nox5 messages are found in breast cancer cell lines.
2. ROS levels are increased in some breast cancer cells lines when compared to control cells.
3. Increased ROS levels correspond to increased tumorigenicity of the cell line.
4. Estradiol increased ROS levels in an estradiol dependent breast cancer cell line.
5. Nox4 protein is expressed in the cell lines.

REPORTABLE OUTCOMES

No reportable outcomes

CONCLUSIONS

We can conclude that ROS does increase in breast cancer cells and this increase correlates with an increase in tumorigenicity of the cell lines. Interestingly, MCF7, which are not very tumorigenic except in the presence of estradiol, show increases in ROS in the presence of estradiol. While the increase in ROS does not correspond to the appearance of any particular Nox isoform (all the breast cell lines tested contain Nox4 and Nox5) this does not rule out the possibility of increased activity of one or both of the isoforms in the breast cancer cell lines. The data that has been obtained is very encouraging and we shall continue to explore the biological function of Nox4 and Nox5 in breast cancer.

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